533 Rec'd PCT/PTO 10 SEP 2001

FORM PTO-1390 (REV 10-95)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

# TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. §371

ATTORNEY'S DOCKET NUMBER

SCH 1821

U.S. APPLICATION NO. (If known, see 37 CFR §1.5)

09/936133

| INTERNATIONAL APPLICATION NO. | INTERNATIONAL FILING DATE | PRIORITY DATE CLAIMED |
|---|---|---|
| PCT/EP00/02005 | 8 MARCH 2000 | 9 MARCH 1999 |

TITLE OF INVENTION

HUMAN NUCLEIC ACID SEQUENCES AND PROTEIN SEQUENCES FROM ENDOTHELIAL CELLS

APPLICANT(S) FOR DO/EO/US

THIERAUCH, Karl-Heinz, et al.

**Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:**

1. ■ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. §371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. §371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. §371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. §371(b) and PCT Articles 22 and 39(1).
4. ■ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ■ A copy of the International Application as filed (35 U.S.C. §371(c)(2))
   - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   - b. ■ has been transmitted by the International Bureau.
   - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ■ A translation of the International Application into English (35 U.S.C. §371(c)(2)).
7. ■ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. §371(c)(3))
   - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   - b. ☐ have been transmitted by the International Bureau.
   - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   - d. ■ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. §371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. §371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371(c)(5)).

**Items 11. to 16. below concern document(s) or information included:**

11. ☐ An Information Disclosure Statement under 37 C.F.R. §§1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. §§3.28 and 3.31 is included.
13. ■ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☐ Other items or information:



(November 1998)

518 Rec'd PCT/PTO 10 SEP 2001

| U.S. APPLICATION NO. (if known, see 37 CFR §1.5) | INTERNATIONAL APPLICATION NO. | ATTORNEY'S DOCKET NUMBER |
|---|---|---|
| 09/936133 | PCT/EP00/02005 | SCH 1821 |

17. ☒ The following fees are submitted:

| | | CALCULATIONS | PTO USE ONLY |
|---|---|---|---|
| **BASIC NATIONAL FEE ( 37 CFR §1.492 (a) (1) - (5)):** | | | |
| Search Report has been prepared by the EPO or JPO | $860.00 | | |
| International preliminary examination fee paid to USPTO (37 CFR §1.482) | $690.00 | | |
| No international preliminary examination fee paid to USPTO (37 CFR §1.482) but international search fee paid to USPTO (37 CFR §1.445(a)(2)) | $710.00 | | |
| Neither international preliminary examination fee (37 CFR §1.482) nor international search fee (37 CFR §1.445(a)(2)) paid to USPTO | $1000.00 | | |
| International preliminary examination fee paid to USPTO (37 CFR §1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) | $100.00 | | |
| **ENTER APPROPRIATE BASIC FEE AMOUNT =** | | $860.00 | |
| Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. §1.492(e)). | | | |

| CLAIMS | NUMBER FILED | NUMBER EXTRA | RATE | | |
|---|---|---|---|---|---|
| Total claims | 40 - 20 = | 20 | x $ 18.00 | $360.00 | |
| Independent claims | 10 - 3 = | 3 | x $ 80.00 | $240.00 | |
| MULTIPLE DEPENDENT CLAIM(S) (if applicable) | | | + $ 270.00 | | |
| **TOTAL OF ABOVE CALCULATIONS =** | | | | $1,460.00 | |
| Reduction of 1/2 for filing by small entity, if applicable. A Verified Small Entity Statement must also be filed (Note 37 C.F.R. §§1.9, 1.27, 1.28). | | | | | |
| **SUBTOTAL =** | | | | $1,460.00 | |
| Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. §1.492(f)). | | | | | |
| **TOTAL NATIONAL FEE =** | | | | $1,460.00 | |
| Fee for recording the enclosed assignment (37 C.F.R. §1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§3.28, 3.31). $40.00 per property. | | | | | |
| **TOTAL FEES ENCLOSED =** | | | | $1,460.00 | |
| | | | | Amount to be refunded: | |
| | | | | charged: | |

a. ■ A check in the amount of $1,460.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 13-3402 in the amount of $ ________ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ■ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 13-3402. A duplicate copy of this sheet is enclosed.

**NOTE: Where an appropriate time limit under 37 C.F.R. §§1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. §1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

SEND ALL CORRESPONDENCE TO: Customer Number 23,599

23599

PATENT TRADEMARK OFFICE

SIGNATURE

Richard J. Traverso

NAME

30,595

REGISTRATION NUMBER

Filed: 7 SEPTEMBER 2001

RJT:kmo

$PCT

JC20 Rec'd PCT/PTO 22 APR 2002

OIPE JC176 APR 22 2002 PATENT & TRADEMARK OFFICE

# IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

| | | |
|---|---|---|
| International Application No. | : | PCT/EP00/02005 |
| International Filing Date | : | 8 MARCH 2000 |
| U.S. Serial No. | : | 09/936,133 |
| Deposit Date U.S. Nat'l Phase | : | 7 SEPTEMBER 2001 |
| Priority Date(s) Claimed | : | 9 MARCH 1999 |
| Applicant(s) | : | THIERAUCH, Karl-Heinz, et al. |

Title: HUMAN NUCLEIC ACID SEQUENCES AND PROTEIN SEQUENCES FROM ENDOTHELIAL CELLS

09936133 .122001

## RESPONSE TO NOTIFICATION OF DEFECTIVE RESPONSE IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

Commissioner for Patents
Box PCT
Washington, D.C. 20231

Sir:

In response to the Notification of Defective Response mailed 11 FEBRUARY 2002, attached is a paper and disk version of the Sequence Listing, a statement affirming that the paper and disk versions are identical, as well as a copy of the Notification.

Applicants request that the time for responding to this action be extended 4 month(s) from the mailing date of the Notification of Missing Requirements to 22 APRIL 2002. A check for the statutory fee or $1,440.00 is enclosed.

The Patent and Trademark Office is authorized to deduct any additional fees from, or credit any overpayments to, counsel's deposit account No. 13-3402, a copy of this paper being attached.

Respectfully submitted,

04/26/2002 MALI11 00000030 09936133
01 FC:118 1440.00 DP

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2200 Clarendon Boulevard, Suite 1400
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Internet Address:zelano@mwzb.com

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

**Atty. Docket No: SCH 1821**

*In re* patent application of

THIERAUCH, KARL-HEINZ *et al.*

Serial No. 09/936,133

Filed: September 7, 2001

For: HUMAN NUCLEIC ACID SEQUENCES AND PROTEIN SEQUENCES FROM ENDOTHELIAL CELLS



STATEMENT TO SUPPORT FILING AND SUBMISSION IN ACCORDANCE WITH 37 C.F.R. §§ 1.821-1.825

Assistant Commissioner for Patents
Washington, D.C. 20231
**Box SEQUENCE**

Sir:

In connection with a Sequence Listing submitted concurrently herewith, the undersigned hereby states that:

1. the submission, filed herewith in accordance with 37 C.F.R. § 1.821(g), does not include new matter;

2. the content of the attached paper copy and the attached computer readable copy of the Sequence Listing, submitted in accordance with 37 C.F.R. § 1.821(c) and (e), respectively, are the same; and

3. all statements made herein of their own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United

States Code and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

Respectfully submitted,

April 19, 2002
Date

James A. Coburn

**HARBOR CONSULTING**
Intellectual Property Services
1500A Lafayette Road
Suite 262
Portsmouth, N.H.
800-318-3021

SEQUENCE LISTING

<110> THIERAUCH, KARL-HEINZ
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HINZMANN, BERND
PILARSKY, CHRISTIAN

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<212> DNA
<213> Homo sapiens

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<212> DNA
<213> Homo sapiens

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<211> 389
<212> DNA
<213> Homo sapiens

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<210> 12
<211> 981
<212> DNA
<213> Homo sapiens

<400> 12
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<210> 13
<211> 401
<212> DNA
<213> Homo sapiens

<400> 13
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<212> DNA
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<211> 280

<212> DNA
<213> Homo sapiens

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<210> 16
<211> 2041
<212> DNA
<213> Homo sapiens

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<210> 18
<211> 2732
<212> DNA
<213> Homo sapiens

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<213> Homo sapiens

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<210> 20
<211> 2361
<212> DNA
<213> Homo sapiens

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<211> 179
<212> DNA
<213> Homo sapiens

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<212> DNA
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<212> DNA
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<212> DNA
<213> Homo sapiens

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<211> 1420

<212> DNA
<213> Homo sapiens

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<211> 689
<212> DNA
<213> Homo sapiens

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<212> DNA
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<210> 28
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<212> DNA
<213> Homo sapiens

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<210> 29
<211> 1775
<212> DNA
<213> Homo sapiens

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<212> DNA
<213> Homo sapiens

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<211> 750
<212> DNA
<213> Homo sapiens

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<210> 32
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<212> DNA
<213> Homo sapiens

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Arg Ala Leu Val Cys Leu Pro Cys Asp Glu Ser Lys Cys Glu Glu Pro
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Arg Asn Arg Pro Gly Ser Ile Val Gln Gly Val Cys Gly Cys Cys Tyr
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Thr Cys Ala Ser Gln Gly Asn Glu Ser Cys Gly Gly Thr Phe Gly Ile
65                  70                  75                  80

Tyr Gly Thr Cys Asp Arg Gly Leu Arg Cys Val Ile Arg Pro Pro Leu
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Asn Gly Asp Ser Leu Thr Glu Tyr Glu Ala Gly Val Cys Glu Asp Glu
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Asn Trp Thr Asp Asp Gln Leu Leu Gly Phe Lys Pro Cys Asn Glu Asn
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Ala Arg Cys Glu Val Gln Phe Ser Pro Arg Cys Pro Glu Asp Ser Val
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Leu Ile Glu Gly Tyr Ala Pro Pro Gly Glu Cys Cys Pro Leu Pro Ser
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Arg Cys Val Cys Asn Pro Ala Gly Cys Leu Arg Lys Val Cys Gln Pro
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Gly Asn Leu Asn Ile Leu Val Ser Lys Ala Ser Gly Lys Pro Gly Glu
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Cys Cys Asp Leu Tyr Glu Cys Lys Pro Val Phe Gly Val Asp Cys Arg
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Thr Val Glu Cys Pro Thr Val Gln Gln Thr Ala Cys Pro Pro Asp Ser
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Tyr Glu Thr Gln Val Arg Leu Thr Ala Asp Gly Cys Cys Thr Leu Pro
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Thr Arg Cys Glu Cys Leu Ser Gly Leu Cys Gly Phe Pro Val Cys Glu
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Val Gly Ser Thr Pro Arg Ile Val Ser Arg Gly Asp Gly Thr Pro Gly
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Lys Cys Cys Asp Val Phe Glu Cys Val Asn Asp Thr Lys Pro Ala Cys
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Glu Cys Cys Pro Val Cys Glu Asp Pro Val Tyr Pro Phe Asn Asn Pro
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Arg Glu Asp Asp Cys Thr Phe Cys Gln Cys Val Asn Gly Glu Arg His
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Asp Lys Tyr Cys Pro Leu Gly Leu Leu Lys Asn Lys His Gly Cys Asp
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Ile Cys Arg Cys Lys Lys Cys Pro Glu Leu Ser Cys Ser Lys Ile Cys
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Phe Ser Glu Ser Cys Pro Ser Val Ser Cys Glu Arg Pro Val Leu Arg
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Lys Gly Gln Cys Cys Pro Tyr Cys Ile Lys Asp Thr Ile Pro Lys Lys
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Val Val Cys His Phe Ser Gly Lys Ala Tyr Ala Asp Glu Glu Arg Trp
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Asp Leu Asp Ser Cys Thr His Cys Tyr Cys Leu Gln Gly Gln Thr Leu
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Cys Ser Thr Val Ser Cys Pro Pro Leu Pro Cys Val Glu Pro Ile Asn
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Val Glu Gly Ser Cys Cys Pro Met Cys Pro Glu Met Tyr Val Pro Glu
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Pro Thr Asn Ile Pro Ile Glu Lys Thr Asn His Arg Gly Glu Val Asp
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Leu Pro Arg Asp Met Gly His Leu Gln Val Asp Tyr Arg Asp Asn Arg
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Leu His Pro Ser Glu Asp Ser Ser Leu Asp Ser Ile Ala Ser Val Val
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Val Pro Ile Ile Ile Cys Leu Ser Ile Ile Ile Ala Phe Leu Phe Ile
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Asn Gln Lys Lys Gln Trp Ile Pro Leu Leu Cys Trp Tyr Arg Thr Pro
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Thr Lys Pro Ser Ser Leu Asn Asn Gln Leu Val Ser Val Asp Cys Lys
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Lys Gly Thr Arg Val Gln Val Asp Ser Ser Gln Arg Met Leu Arg Ile
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aagggacacc gtgt 134

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<211> 644
<212> DNA
<213> Homo sapiens

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<210> 40
<211> 657
<212> DNA
<213> Homo sapiens

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<210> 41
<211> 1328
<212> DNA
<213> Homo sapiens

<400> 41
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<213> Homo sapiens

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<211> 956
<212> DNA
<213> Homo sapiens

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<211> 536
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<213> Homo sapiens

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<210> 45
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<212> DNA
<213> Homo sapiens

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<211> 169
<212> DNA
<213> Homo sapiens

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<210> 47
<211> 769
<212> DNA
<213> Homo sapiens

<400> 47
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<211> 2529
<212> DNA
<213> Homo sapiens

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<211> 1552

<212> DNA
<213> Homo sapiens

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<212> DNA
<213> Homo sapiens

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<211> 338
<212> DNA
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

**Atty. Docket No: SCH 1821**

*In re* patent application of

THIERAUCH, KARL-HEINZ *et al.*

Serial No. 09/936,133

Filed: September 7, 2001

For: HUMAN NUCLEIC ACID SEQUENCES AND PROTEIN SEQUENCES FROM ENDOTHELIAL CELLS

STATEMENT TO SUPPORT FILING AND SUBMISSION IN ACCORDANCE WITH 37 C.F.R. §§ 1.821-1.825

Assistant Commissioner for Patents
Washington, D.C. 20231
**Box SEQUENCE**

Sir:

In connection with a Sequence Listing submitted concurrently herewith, the undersigned hereby states that:

1. the submission, filed herewith in accordance with 37 C.F.R. § 1.821(g), does not include new matter;

2. the content of the attached paper copy and the attached computer readable copy of the Sequence Listing, submitted in accordance with 37 C.F.R. § 1.821(c) and (e), respectively, are the same; and

3. all statements made herein of their own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United

States Code and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

Respectfully submitted,

April 19, 2002
Date

James A. Coburn

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800-318-3021

SEQUENCE LISTING

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<212> DNA
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<213> Homo sapiens

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<213> Homo sapiens

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<211> 2361
<212> DNA
<213> Homo sapiens

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<212> DNA
<213> Homo sapiens

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tttataagat caacgtgaca agttgaagga aattgaaagg aaaaaattag aactaatgc 179

<210> 22
<211> 905
<212> DNA
<213> Homo sapiens

<400> 22
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<212> DNA
<213> Homo sapiens

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<211> 1626
<212> DNA
<213> Homo sapiens

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<211> 1420

<212> DNA
<213> Homo sapiens

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<211> 689
<212> DNA
<213> Homo sapiens

<400> 26
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<211> 471
<212> DNA
<213> Homo sapiens

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<210> 28
<211> 929
<212> DNA
<213> Homo sapiens

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<212> PRT
<213> Homo sapiens

<400> 35

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Arg Ala Leu Val Cys Leu Pro Cys Asp Glu Ser Lys Cys Glu Glu Pro
        35                  40                  45

Arg Asn Arg Pro Gly Ser Ile Val Gln Gly Val Cys Gly Cys Cys Tyr
    50                  55                  60

Thr Cys Ala Ser Gln Gly Asn Glu Ser Cys Gly Gly Thr Phe Gly Ile
65                  70                  75                  80

Tyr Gly Thr Cys Asp Arg Gly Leu Arg Cys Val Ile Arg Pro Pro Leu
                85                  90                  95

Asn Gly Asp Ser Leu Thr Glu Tyr Glu Ala Gly Val Cys Glu Asp Glu
            100                 105                 110

Asn Trp Thr Asp Asp Gln Leu Leu Gly Phe Lys Pro Cys Asn Glu Asn
        115                 120                 125
```

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Leu Ile Ala Gly Cys Asn Ile Ile Asn Gly Lys Cys Glu Cys Asn Thr
    130                 135                 140

Ile Arg Thr Cys Ser Asn Pro Phe Glu Phe Pro Ser Gln Asp Met Cys
145                 150                 155                 160

Leu Ser Ala Leu Lys Arg Ile Glu Glu Glu Lys Pro Asp Cys Ser Lys
                165                 170                 175

Ala Arg Cys Glu Val Gln Phe Ser Pro Arg Cys Pro Glu Asp Ser Val
            180                 185                 190

Leu Ile Glu Gly Tyr Ala Pro Pro Gly Glu Cys Cys Pro Leu Pro Ser
        195                 200                 205

Arg Cys Val Cys Asn Pro Ala Gly Cys Leu Arg Lys Val Cys Gln Pro
    210                 215                 220

Gly Asn Leu Asn Ile Leu Val Ser Lys Ala Ser Gly Lys Pro Gly Glu
225                 230                 235                 240

Cys Cys Asp Leu Tyr Glu Cys Lys Pro Val Phe Gly Val Asp Cys Arg
                245                 250                 255

Thr Val Glu Cys Pro Thr Val Gln Gln Thr Ala Cys Pro Pro Asp Ser
            260                 265                 270

Tyr Glu Thr Gln Val Arg Leu Thr Ala Asp Gly Cys Cys Thr Leu Pro
        275                 280                 285

Thr Arg Cys Glu Cys Leu Ser Gly Leu Cys Gly Phe Pro Val Cys Glu
    290                 295                 300

Val Gly Ser Thr Pro Arg Ile Val Ser Arg Gly Asp Gly Thr Pro Gly
305                 310                 315                 320

Lys Cys Cys Asp Val Phe Glu Cys Val Asn Asp Thr Lys Pro Ala Cys
                325                 330                 335

Val Phe Asn Asn Val Glu Tyr Tyr Asp Gly Asp Met Phe Arg Met Asp
            340                 345                 350

Asn Cys Arg Phe Cys Arg Cys Gln Gly Gly Val Ala Ile Cys Phe Thr
        355                 360                 365

Ala Gln Cys Gly Glu Ile Asn Cys Glu Arg Tyr Tyr Val Pro Glu Gly
    370                 375                 380

Glu Cys Cys Pro Val Cys Glu Asp Pro Val Tyr Pro Phe Asn Asn Pro
385                 390                 395                 400

Ala Gly Cys Tyr Ala Asn Gly Leu Ile Leu Ala His Gly Asp Arg Trp
                405                 410                 415

Arg Glu Asp Asp Cys Thr Phe Cys Gln Cys Val Asn Gly Glu Arg His
            420                 425                 430
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Cys Val Ala Thr Val Cys Gly Gln Thr Cys Thr Asn Pro Val Lys Val
        435                 440                 445

Pro Gly Glu Cys Cys Pro Val Cys Glu Glu Pro Thr Ile Ile Thr Val
    450                 455                 460

Asp Pro Pro Ala Cys Gly Glu Leu Ser Asn Cys Thr Leu Thr Arg Lys
465                 470                 475                 480

Asp Cys Ile Asn Gly Phe Lys Arg Asp His Asn Gly Cys Arg Thr Cys
                485                 490                 495

Gln Cys Ile Asn Thr Gln Glu Leu Cys Ser Glu Arg Lys Gln Gly Cys
            500                 505                 510

Thr Leu Asn Cys Pro Phe Gly Phe Leu Thr Asp Ala Gln Asn Cys Glu
        515                 520                 525

Ile Cys Glu Cys Arg Pro Arg Pro Lys Lys Cys Arg Pro Ile Ile Cys
    530                 535                 540

Asp Lys Tyr Cys Pro Leu Gly Leu Leu Lys Asn Lys His Gly Cys Asp
545                 550                 555                 560

Ile Cys Arg Cys Lys Lys Cys Pro Glu Leu Ser Cys Ser Lys Ile Cys
                565                 570                 575

Pro Leu Gly Phe Gln Gln Asp Ser His Gly Cys Leu Ile Cys Lys Cys
            580                 585                 590

Arg Glu Ala Ser Ala Ser Ala Gly Pro Pro Ile Leu Ser Gly Thr Cys
        595                 600                 605

Leu Thr Val Asp Gly His His His Lys Asn Glu Glu Ser Trp His Asp
    610                 615                 620

Gly Cys Arg Glu Cys Tyr Cys Leu Asn Gly Arg Glu Met Cys Ala Leu
625                 630                 635                 640

Ile Thr Cys Pro Val Pro Ala Cys Gly Asn Pro Thr Ile His Pro Gly
                645                 650                 655

Gln Cys Cys Pro Ser Cys Ala Asp Asp Phe Val Val Gln Lys Pro Glu
            660                 665                 670

Leu Ser Thr Pro Ser Ile Cys His Ala Pro Gly Gly Glu Tyr Phe Val
        675                 680                 685

Glu Gly Glu Thr Trp Asn Ile Asp Ser Cys Thr Gln Cys Thr Cys His
    690                 695                 700

Ser Gly Arg Val Leu Cys Glu Thr Glu Val Cys Pro Pro Leu Leu Cys
705                 710                 715                 720

Gln Asn Pro Ser Arg Thr Gln Asp Ser Cys Cys Pro Gln Cys Thr Asp
                725                 730                 735
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Gln Pro Phe Arg Pro Ser Leu Ser Arg Asn Asn Ser Val Pro Asn Tyr
            740                 745                 750

Cys Lys Asn Asp Glu Gly Asp Ile Phe Leu Ala Ala Glu Ser Trp Lys
        755                 760                 765

Pro Asp Val Cys Thr Ser Cys Ile Cys Ile Asp Ser Val Ile Ser Cys
    770                 775                 780

Phe Ser Glu Ser Cys Pro Ser Val Ser Cys Glu Arg Pro Val Leu Arg
785                 790                 795                 800

Lys Gly Gln Cys Cys Pro Tyr Cys Ile Lys Asp Thr Ile Pro Lys Lys
                805                 810                 815

Val Val Cys His Phe Ser Gly Lys Ala Tyr Ala Asp Glu Glu Arg Trp
            820                 825                 830

Asp Leu Asp Ser Cys Thr His Cys Tyr Cys Leu Gln Gly Gln Thr Leu
        835                 840                 845

Cys Ser Thr Val Ser Cys Pro Pro Leu Pro Cys Val Glu Pro Ile Asn
    850                 855                 860

Val Glu Gly Ser Cys Cys Pro Met Cys Pro Glu Met Tyr Val Pro Glu
865                 870                 875                 880

Pro Thr Asn Ile Pro Ile Glu Lys Thr Asn His Arg Gly Glu Val Asp
                885                 890                 895

Leu Glu Val Pro Leu Trp Pro Thr Pro Ser Glu Asn Asp Ile Val His
            900                 905                 910

Leu Pro Arg Asp Met Gly His Leu Gln Val Asp Tyr Arg Asp Asn Arg
        915                 920                 925

Leu His Pro Ser Glu Asp Ser Ser Leu Asp Ser Ile Ala Ser Val Val
    930                 935                 940

Val Pro Ile Ile Ile Cys Leu Ser Ile Ile Ile Ala Phe Leu Phe Ile
945                 950                 955                 960

Asn Gln Lys Lys Gln Trp Ile Pro Leu Leu Cys Trp Tyr Arg Thr Pro
                965                 970                 975

Thr Lys Pro Ser Ser Leu Asn Asn Gln Leu Val Ser Val Asp Cys Lys
            980                 985                 990

Lys Gly Thr Arg Val Gln Val Asp Ser Ser Gln Arg Met Leu Arg Ile
        995                 1000                1005

Ala Glu Pro Asp Ala Arg Phe Ser Gly Phe Tyr Ser Met Gln Lys Gln
    1010                1015                1020

Asn His Leu Gln Ala Asp Asn Phe Tyr Gln Thr Val
1025                1030                1035
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<212> DNA
<213> Homo sapiens

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agaagtccag agggatcagc cccagaacac accctcctcc ccgggacgcc gcagctttct 180
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gtcagtgtgg agtagacggt ttcctccacc cagggttgac tcagggggat gatctgggtc 480
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cccccatcca cctaacaggg tggccacagg gattactgag ggttaagacc ttagaactgg 660
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<213> Homo sapiens

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tctccaaaaa aaccttgaaa tgaagaaggc cacccttaaa atacttcaga ggctgaaaat 180
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gcccagtaaa accaaagtca gggtaaccaa tgtcatttta caaaatgtta aaaccctaat 300
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agaagcaagg aaagcattct taattctacc atcct 395

<210> 38
<211> 134
<212> DNA
<213> Homo sapiens

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aagggacacc gtgt 134

<210> 39
<211> 644
<212> DNA
<213> Homo sapiens

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<211> 657
<212> DNA
<213> Homo sapiens

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<211> 1328
<212> DNA
<213> Homo sapiens

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<211> 987
<212> DNA
<213> Homo sapiens

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<211> 956
<212> DNA
<213> Homo sapiens

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<211> 536
<212> DNA
<213> Homo sapiens

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<210> 45
<211> 1630

<212> DNA
<213> Homo sapiens

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<211> 169
<212> DNA
<213> Homo sapiens

<400> 46
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<211> 769
<212> DNA
<213> Homo sapiens

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<210> 48
<211> 2529
<212> DNA
<213> Homo sapiens

<400> 48

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<211> 1552

<212> DNA
<213> Homo sapiens

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<211> 921
<212> DNA
<213> Homo sapiens

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<211> 338
<212> DNA
<213> Homo sapiens

<400> 51
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catgttctgt cttcatttcc tcatgttcaa cccaccctgt cttagctacc acccctcagt 180
aacgacctag cctgggtaga aacaaatgtc agcatgatac catactcaat gatccttcgt 240
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gtaagaaact ttctttcttg aattcttggt tctcttgg                         338
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<210> 52
<211> 1191
<212> DNA
<213> Homo sapiens

<400> 52
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cctggaaaat aatgaattgg gtaaggaaca gttaataaga aaatgtgcct tgctaactgt 180
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<210> 53
<211> 1200
<212> DNA
<213> Homo sapiens

<400> 53
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<211> 989
<212> DNA
<213> Homo sapiens

<400> 54
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<211> 250
<212> DNA
<213> Homo sapiens

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<210> 56
<211> 2270
<212> DNA
<213> Homo sapiens

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<212> DNA
<213> Homo sapiens

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09/936133
518 Rec'd PCT/PTO 10 SEP 2001

**IN THE UNITED STATES DESIGNATED/ELECTED OFFICE**

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| International Application No. | : | PCT/EP00/02005 |
| International Filing Date | : | 8 MARCH 2000 |
| Priority Date(s) Claimed | : | 9 MARCH 1999 |
| Applicant(s) (DO/EO/US) | : | THIERAUCH, Karl-Heinz, et al. |

Title: HUMAN NUCLEIC ACID AND PROTEIN SEQUENCES OBTAINED FROM ENDOTHELIAL CELLS

**PRELIMINARY AMENDMENT**

Commissioner for Patents
Washington, D.C. 20231

SIR:

Prior to calculating the national fee, and prior to examination in the National Phase of the above-identified International application, please amend as follows:

IN THE CLAIMS:

5. (Amended) A nucleic acid sequence according to claim 1, wherein it has 90% homology to a human nucleic acid sequence.

6. (Amended) A nucleic acid sequence according to claim 1, wherein it has 95% homology to a human nucleic acid sequence.

7. (Amended) A nucleic acid sequence comprising a portion of the nucleic acid sequences named in claim 1, in such a sufficient amount that they hybridize with the sequences according to claim 1.

8. (Amended) A nucleic acid sequence according to claim 1, wherein the size of the fragment has a length of at least 50 to 3000 bp.

9. (Amended) A nucleic acid sequence according to claim 1 , wherein the size of the fragment has a length of at least 150 to 2800 bp.

10. (Amended) A nucleic acid sequence according to claim 1, wherein the size of the fragment has a length of at least a 150 to 2600 bp.

11. (Amended) A nucleic acid sequence according to claim 1, which codes at least one partial sequence of a bioactive polypeptide.

12. (Amended) An expression cassette, comprising a nucleic acid fragment or a sequence according to claim 1, together with at least one control or regulatory sequence.

14. (Amended) An expression cassette according to claim 12, wherein the DNA sequences located on the cassette code a fusion protein, which comprises a known protein and a bioactive polypeptide fragment.

15. (Amended) Use of nucleic acid sequences according to claim 1 for producing full-length genes.

17. (Amended) Host cell, containing as the heterologous part of its expressible genetic information a nucleic acid fragment according to claim 1.

19. (Amended) Host cell according to claim 17, wherein the prokaryotic cell system is E. coli, and the eukaryotic cell system is an animal, human or yeast cell system.

20. (Amended) A process for the production of a polypeptide or a fragment, wherein the host cells according to claim 17 are cultivated.

27. (Amended) Use of polypeptide sequences according to claim 23 as tools for finding active ingredients against angiogenetic diseases.

30. (Amended) Use of polypeptide sequences according to claim 23 as pharmaceutical agents in gene therapy for treatment of angiogenetic diseases.

31. (Amended) Use of polypeptide sequences according to claim 23 for the production of a pharmaceutical agent for treatment of angiogenetic diseases.

32. (Amended) Pharmaceutical agent, containing at least one polypeptide sequence according to claim 23.

33. (Amended) A nucleic acid sequence according to claim 1, wherein it is a genomic sequence.

34. (Amended) A nucleic acid sequence according to claim 1, wherein it is an mRNA sequence.

38. (Amended) Use of the nucleic acid sequences according to claim 1 and the peptides expressed by one of nucleic acid sequences Seq. ID No. 1 to Seq. ID No. 59, either alone or in a formulation as a pharmaceutical agent for treatment of psoriasis, arthritis, such as rheumatoid arthritis, hemangioma, angiofibroma, eye diseases, such as diabetic retinopathy, neovascular glaucoma, nephropathies, such as glomerulonephritis, diabetic nephropathy, malignant nephrosclerosis, thrombic microangiopathic syndrome, transplant rejections and glomerupathy, fibrotic diseases, such as cirrhosis of the liver, mesangial cell proliferative diseases, arteriosclerosis and injuries of the nerve tissue.

## REMARKS

The purpose of this Preliminary Amendment is to eliminate multiple dependent claims in order to avoid the additional fee. Applicants reserve the right to reintroduce claims to canceled combined subject matter.

Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached pages are captioned "**Version With Markings to Show Changes Made**".

Respectfully submitted,

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AJZ(RJT):jmm

FILED: 7 SEPTEMBER 2001

VERSION WITH MARKINGS TO SHOW CHANGES MADE

Claims 5-12, 14-15, 17, 19-20, 27, 30-34 and 38 have been amended as follows:

5. (Amended) A nucleic acid sequence according to claim~~s~~ 1~~ to 4~~, wherein it has 90% homology to a human nucleic acid sequence.

6. (Amended) A nucleic acid sequence according to claim~~s~~ 1~~ to 4~~, wherein it has 95% homology to a human nucleic acid sequence.

7. (Amended) A nucleic acid sequence comprising a portion of the nucleic acid sequences named in claim~~s~~ 1~~ to 6~~, in such a sufficient amount that they hybridize with the sequences according to claim~~s~~ 1~~ to 6~~.

8. (Amended) A nucleic acid sequence according to claim~~s~~ 1~~ to 7~~, wherein the size of the fragment has a length of at least 50 to 3000 bp.

9. (Amended) A nucleic acid sequence according to claim~~s~~ 1 ~~to 7;~~ wherein the size of the fragment has a length of at least 150 to 2800 bp.

10. (Amended) A nucleic acid sequence according to claim~~s~~ 1~~ to 7~~, wherein the size of the fragment has a length of at least a 150 to 2600 bp.

11. (Amended) A nucleic acid sequence according to ~~one of~~ claims 1~~ to 10~~, which codes at least one partial sequence of a bioactive polypeptide.

12. (Amended) An expression cassette, comprising a nucleic acid fragment or a sequence according to ~~one of~~ claim~~s~~ 1~~ to 10~~, together with at least one control or regulatory sequence.

14. (Amended) An expression cassette according to ~~one of~~ claim~~s~~ 12~~ and 13~~, wherein the DNA sequences located on the cassette code a fusion protein, which comprises a

known protein and a bioactive polypeptide fragment.

15. (Amended) Use of nucleic acid sequences according to claim~~s 1 to 11~~ 1 for producing full-length genes.

17. (Amended) Host cell, containing as the heterologous part of its expressible genetic information a nucleic acid fragment according to ~~one of~~ claim~~s 1 to 11~~ 1.

19. (Amended) Host cell according to ~~one of~~ claim~~s~~ 17 ~~or 18~~, wherein the prokaryotic cell system is E. coli, and the eukaryotic cell system is an animal, human or yeast cell system.

20. (Amended) A process for the production of a polypeptide or a fragment, wherein the host cells according to claim~~s~~ 17 ~~to 19~~ are cultivated.

27. (Amended) Use of polypeptide sequences according to claim~~s~~ 23 ~~to 26~~ as tools for finding active ingredients against angiogenetic diseases.

30. (Amended) Use of polypeptide sequences according to claim~~s~~ 23 ~~to 26~~ as pharmaceutical agents in gene therapy for treatment of angiogenetic diseases.

31. (Amended) Use of polypeptide sequences according to claim~~s~~ 23 ~~to 26~~ for the production of a pharmaceutical agent for treatment of angiogenetic diseases.

32. (Amended) Pharmaceutical agent, containing at least one polypeptide sequence according to claim~~s~~ 23 ~~to 26~~.

33. (Amended) A nucleic acid sequence according to claim~~s 1 to 11~~ 1, wherein it is a genomic sequence.

34. (Amended) A nucleic acid sequence according to claim~~s 1 to 11~~ 1, wherein it is an mRNA sequence.

38. (Amended) Use of the nucleic acid sequences according to claim~~s 1 to 11~~ 1 and the peptides ~~according~~expressed by one of nucleic acid sequences Seq. ID No. 1 to ~~claims 23 to 26~~Seq. ID No. 59, either alone or in a formulation as a pharmaceutical agent for treatment of psoriasis, arthritis, such as rheumatoid arthritis, hemangioma, angiofibroma, eye diseases, such as diabetic retinopathy, neovascular glaucoma, nephropathies, such as glomerulonephritis, diabetic nephropathy, malignant nephrosclerosis, thrombic microangiopathic syndrome, transplant rejections and glomerupathy, fibrotic diseases, such as cirrhosis of the liver, mesangial cell proliferative diseases, arteriosclerosis and injuries of the nerve tissue.

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<400> 11

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<400> 16

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<400> 19

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<211> 2361
<212> DNA
<213> homo sapiens

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915 920 925
Leu His Pro Ser Glu Asp Ser Ser Leu Asp Ser Ile Ala Ser Val Val
930 935 940
Val Pro Ile Ile Ile Cys Leu Ser Ile Ile Ile Ala Phe Leu Phe Ile
945 950 955 960
Asn Gln Lys Lys Gln Trp Ile Pro Leu Leu Cys Trp Tyr Arg Thr Pro
965 970 975

Thr Lys Pro Ser Ser Leu Asn Asn Gln Leu Val Ser Val Asp Cys Lys
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Lys Gly Thr Arg Val Gln Val Asp Ser Ser Gln Arg Met Leu Arg Ile
995 1000 1005
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1010 1015 1020
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<400> 36

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<400> 37

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<211> 134
<212> DNA
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<400> 38

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<210> 39
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<400> 39

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<211> 657
<212> DNA
<213> homo sapiens

<400> 40

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<212> DNA
<213> homo sapiens

<400> 41

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<212> DNA
<213> homo sapiens

<400> 42

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<210> 43
<211> 956
<212> DNA
<213> homo sapiens

<400> 43

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<211> 536
<212> DNA
<213> homo sapiens

<400> 44

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<210> 45
<211> 1630
<212> DNA

<213> homo sapiens

<400> 45

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<212> DNA
<213> homo sapiens

<400> 46

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<210> 47
<211> 769
<212> DNA
<213> homo sapiens

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<212> DNA
<213> homo sapiens

<400> 49

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# Human Nucleic Acid Sequences and Protein Sequences from Endothelial Cells

The invention relates to nucleic acid sequences -- mRNA, cDNA, genomic sequences -- from tissue of human endothelial cells, which code for gene products or portions thereof, and their use. In addition, the invention relates to the polypeptides that can be obtained by way of the sequences and their use.

Angiogenesis is a process that can be observed in the adult living creature in the cyclic processes of reproduction in the female, in wound healing and in various pathological situations, such as, e.g., tumor growth, rheumatic diseases, endometriosis, in the case of collateral formation in the heart and in the periphery, etc.

Persistent angiogenesis can be the cause of various diseases, such as psoriasis, arthritis, such as rheumatoid arthritis, hemangioma, angiofibroma, eye diseases, such as diabetic retinopathy, neovascular glaucoma, nephropathies, such as glomerulonephritis, diabetic nephropathy, malignant nephrosclerosis, thrombic microangiopathic syndrome, transplant rejections and glomerulopathy, fibrotic diseases, such as cirrhosis of the liver, mesangial cell proliferative diseases, and arteriosclerosis or can lead to an aggravation of these diseases.

If it were possible to induce or to inhibit angiogenesis, it would be possible to ensure thorough treatment of several diseases. To this end, the genes or the nucleic acid sequences that are relevant to the angiogenesis had to become known.

It was not previously known which genes or nucleic acid sequences or portions thereof are angiogenesis-relevant.

Nucleic acid sequences could now be found that are angiogenesis-relevant.

These sequences either have not yet been described or they are known only as nucleic acid sequences from rodents, but without reference to angiogenesis. Additional sequences are described as human genes or portions thereof, but not in reference to possible angiogenesis-relevant properties.

In the search for angiogenesis-relevant genes, endothelial cells were obtained from the foreskins of adults that were cultivated in two different ways:

a) in a rat-tail collagen matrix in subconfluent density and

b) in a gel that consists of an extracellular matrix (matrigel).

Under culture type a), the cells form the standard cobblestone-like monolayer.

Under culture type b), the cells form netlike structures with tubular entities.

Cell culture type a) represents an early angiogenesis state with a first and foremost proliferative phenotype.

Cell culture type b) represents a model for a later phase of angiogenesis, in which the differentiation of the endothelial cells leads to a formation of hose-shaped structures. These structures are a requirement for a blood flow that is separated from the tissue surface.

mRNA is isolated from both cell culture types, transcribed into cDNA and cut with a restriction endonuclease into fragments measuring 200 to 1500 bp. By means of a subtractive PCR technique, the fragments that occur differentially in both states were amplified. They were incorporated into vectors and cloned. The clones were first sequenced, and then their sequences were completed with bioinformatory techniques.

With the aid of a quantitative PCR technique that is described in the literature (Pilarsky et al., 1998, see Test Description), it was first examined whether the genes are expressed differentially in the two culture states. For standardization, the expression of the 23 kDalton protein (see Test Description) was used as an internal marker. In the differential expression, ratios of 2- to 7-fold occurred.

The nucleic acid sequences Seq. ID No. 1 to Seq. ID No. 59 that play a role in angiogenesis as candidate genes could now be found.

The invention thus relates to nucleic acid sequences that code a gene product or a portion thereof, comprising

a) a nucleic acid sequence that is selected from the group of nucleic acid sequences Seq. ID No. 1 to Seq. ID No. 59

b) an allelic variation of the nucleic acid sequences named under a)

or

c) a nucleic acid sequence that is complementary to the nucleic acid sequences named under a) or b).

In addition, the invention relates to nucleic acid sequences according to one of sequences Seq. ID No. 1 to Seq. ID No. 59 or a complementary or allelic variant thereof and the nucleic acid sequences thereof, which have 90% to 95% homology to a human nucleic acid sequence.

The invention also relates to nucleic acid sequences Seq. ID No. 1 to Seq. ID No. 59, which are expressed elevated in endothelial cell tissue.

The invention further relates to nucleic acid sequences comprising a portion of the above-mentioned nucleic acid sequences in such a sufficient amount that they hybridize with sequences Seq. ID No. 1 to Seq. ID No. 59.

The nucleic acid sequences according to the invention generally have a length of at least 50 to 3000 bp, preferably a length of at least 150 to 2800 bp, especially preferably a length of 150 to 2600 bp.

With the sequences Seq. ID No. 1 to Seq. ID No. 59 according to the invention, expression cassettes can also be built using current process practice, whereby on the cassette at least one of the nucleic acid sequences according to the invention is combined with at least one control or regulatory sequence that is generally known to one skilled in the art, such as, e.g., a

suitable promoter. The sequences according to the invention can be inserted in a sense or antisense orientation.

A large number of expression cassettes or vectors and promoters which can be used are known in the literature.

Expression cassettes or vectors are defined as:

1. bacterial, such as, e.g., phagescript, pBs, $\phi$X174, pBluescript SK, pBs KS, pNH8a, pNH16a, pNH18a, pNH46a (Stratagene), pTrc99A, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia),

2. eukaryotic, such as, e.g., pWLneo, pSV2cat, pOG44, pXT1, pSG (Stratagene), pSVK3, pBPV, pMSG, pSVL (Pharmacia).

Control or regulatory sequences are defined as suitable promoters. Here, two preferred vectors are the pKK232-8 and the PCM7 vector. In particular, the following promoters are intended: lacI, lacZ, T3, T7, gpt, lambda $P_R$, trc, CMV, HSV thymidine-kinase, SV40, LTRs from retrovirus and mouse metallothionein-I.

The DNA sequences located on the expression cassette can code a fusion protein that comprises a known protein and a bioactive polypeptide fragment.

The expression cassettes are likewise the subject matter of this invention.

The nucleic acid sequences according to the invention can also be used to produce full-length genes. The genes that can be obtained are likewise the subject matter of this invention.

The invention also relates to the use of the nucleic acid sequences according to the invention and the gene fragments that can be obtained from use.

The nucleic acid sequences according to the invention can be moved with suitable vectors into host cells, in which, as the heterologous part, the genetic information that is contained on the nucleic acid sequences and that is expressed is located.

The host cells containing the nucleic acid sequences are likewise the subject matter of this invention.

Suitable host cells are, e.g., prokaryotic cell systems such as E. coli or eukaryotic cell systems such as animal or human cells or yeasts.

The nucleic acid sequences according to the invention can be used in the sense or antisense form.

Production of polypeptides or their fragments is done by cultivation of the host cells according to current cultivation methods and subsequent isolation and purification of the peptides or fragments, likewise using current processes.

The invention further relates to nucleic acid sequences, which code at least a partial sequence of a bioactive polypeptide.

This invention further relates to polypeptide partial sequences, so-called ORF (open-reading-frame)-peptides that are expressed by the inventive partial sequences.

The invention further relates to the polypeptide sequences that have at least 80% homology, especially 90% homology to the polypeptides.

The invention also relates to antibodies that are directed against a polypeptide or a fragment and that are coded by the nucleic acid sequences Seq. ID No. 1 to Seq. ID No. 59 according to the invention.

Antibodies are defined especially as monoclonal antibodies.

The polypeptides that are coded by the nucleic acid sequences according to the invention can also be used as tools for finding active ingredients in the case of angiogenic diseases, which is likewise the subject matter of this invention.

Likewise the subject matter of this invention is the use of nucleic acid sequences according to sequences Seq. ID No. 1 to Seq. ID No. 59 for expression of polypeptides, which can be used as tools for finding active ingredients against angiogenetic diseases.

The invention also relates to the use of the polypeptides expressed by the nucleic acid sequences Seq. ID No. 1 to Seq. ID No. 59 according to the invention as pharmaceutical agents in gene therapy for the treatment of angiogenic diseases, or for the production of a pharmaceutical agent for treating angiogenic diseases.

The nucleic acids according to the invention or the proteins that are expressed by way of these nucleic acids can thus be used either alone or in a formulation as a pharmaceutical agent for treatment of psoriasis, arthritis, such as rheumatoid arthritis, hemangioma, angiofibroma, eye diseases, such as diabetic retinopathy, neovascular glaucoma, nephropathies, such as glomerulonephritis, diabetic nephropathy, malignant

nephrosclerosis, thrombic microangiopathic syndrome, transplant rejections and glomerulopathy, fibrotic diseases, such as cirrhosis of the liver, mesangial cell proliferative diseases, arteriosclerosis and injuries to the nerve tissue.

The invention also relates to pharmaceutical agents that contain at least one polypeptide sequence that are expressed by the nucleic acid sequences Seq. ID No. 1 to Seq. ID No. 59 according to the invention.

The nucleic acid sequences found according to the invention can also be genomic or mRNA sequences.

The invention also relates to genomic genes, their promotors, enhancers, silencers, Exon structure, Intron structure and their splice variants that can be obtained from cDNAs of sequences Seq. ID No. 1 to Seq. ID No. 59, and their use together with suitable regulatory elements, such as suitable promoters and/or enhancers.

With the nucleic acids according to the invention (cDNA sequences), genomic BAC, PAC and Cosmid libraries are screened and specific human clones are isolated via complementary base pairing (hybridization). The thus isolated BAC, PAC and Cosmid clones are hybridized using fluorescence-in-situ hybridization on metaphase chromosomes and the corresponding chromosome sections on which the corresponding genomic genes lie are identified. BAC, PAC and Cosmid clones are sequenced in order to clarify the corresponding genomic genes in their complete structure (promoters, enhancers, silencers, Exons and Introns). BAC, PAC

and Cosmid clones can be used as independent molecules for gene transfer.

The invention also relates to BAC, PAC and Cosmid clones containing functional genes and their chromosomal localization according to sequences Seq. ID No. 1 to Seq. ID No. 59 for use as vehicles for gene transfer.

**Meanings of Technical Terms and Abbreviations**

| | |
|---|---|
| Nucleic acids = | Nucleic acids in this invention are defined as: mRNA, partial cDNA, full-length cDNA and genomic genes (chromosomes). |
| ORF = | Open Reading Frame, a defined sequence of amino acids that can be derived from the cDNA sequence. |

The following examples explain the production of the nucleic acid sequences according to the invention without limiting the invention to these examples and nucleic acid sequences.

**Example 1**

**1. Search for Angiogenesis-Relevant Candidate Genes**

**1.1 Cells That Are Used**

Primary, human, microvascular endothelial cells (MVEC) were prepared from human foreskins and selected by means of biotinylated anti CD31 (PECAM) antibodies (reference).

Culture conditions: 37°C, 5% $CO_2$

Medium: M199, 10% FCS, 10% human serum, 6 μg/ml of ECGF, 1 mmol of sodium pyruvate, 3 U/ml of heparin, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 1 x non-essential amino acids.

**1.2 Cultivation and RNA Preparation**

For culture type a), the cells are cultivated on plastic that is coated with collagen I. For culture type b), the cells are broken off on a gel that consists of extracellular matrix proteins. The matrigel that is used in this case (Becton Dickinson) was diluted 1 to 1 with M199 medium, poured into the culture vessel used in the cold state (60 $\mu l/cm^2$) and gelled at 37°C for 30 minutes. Then, the cells were broken off.

For culture types a) and b), MVEC in a density of $2x10^4/cm^2$ were broken off and incubated for 7 hours at 37°C, 5% $CO_2$.

The total RNA preparation was performed according to the guanidinium thiocyanate method with subsequent centrifuging through a cesium chloride cushion (Sambrook, J.; Fritsch, E. F.; and Maniatis, T.; 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press). The polyA$^+$ RNA selection was performed by way of oligo (dT)-cellulose columns (mRNA Purification Kit, Pharmacia Biotech).

### 1.3 Adjustment of Subtractive cDNA-Banks

The subtraction was performed according to the method of Diatchenko et al. (Proc. Natl. Acad. Sci. U.S.A., 1996, June 11, 93:6025-30) with the aid of the PCR-select cDNA subtraction kit.

The polyA + RNA, which contains the target sequences, is referred to as tester, and the polyA + RNA that is to be drawn therefrom is referred to as driver.

Two subtractions were performed, whereby polyA + RNA of culture type a) and polyA + RNA of culture type (b) were each used once as tester. The following test description shows only one subtraction by way of example.

### 1.4 Synthesis of Double-Strand cDNA (ds cDNA)

A double-strand cDNA synthesis is performed both for the test and for the driver.

**1. Strand Synthesis**

The strand synthesis is performed with the following batch:

| | |
|---|---|
| polyA + RNA | 2 $\mu$g |
| cDNA-synthesis primer (10 $\mu$M) | 1 $\mu$l |
| water | add 5 $\mu$l |

The reactions are incubated for 2 minutes at 70°C and then for 2 minutes on ice.

The following was added to each reaction:

5 x first-strand buffer (250 mmol of tris-HCL, pH 8, 330 mmol of Mg-chloride, 375 mmol of KCl)

2 $\mu$l

10 mmol of dNTP

1 $\mu$l

water

1 $\mu$l

MMLV reverse transcriptase (200 U/$\mu$l)

1 $\mu$l

The reactions were incubated for 90 minutes at 42°C and then for 2 minutes on ice.

**2. Strand synthesis**

The second strand synthesis was performed with the following batch:

| | |
|---|---|
| 1st Strand synthesis | 10 $\mu$l |
| water | 48.4 $\mu$l |

5x second-strand buffer (500 mmol of KCL, 50 mmol of ammonium sulfate, 25 mmol of Mg-chloride, 0.75 mmol of ß-NAD, 100 mmol of tris-HCL, pH 7.5, 0.25 mg/ml of BSA) 16 μl

10 mmol of dNTP 1.6 μl

20x second-strand enzyme cocktail (DNA polymerase 1 6 U/μl of Rnase H 0.2 U/μl, **E. coli** DNA ligase 1.2 U/μl) 4 μl

The reactions were incubated for 2 hours at 16°C.

T4 DNA polymerase was added as follows to each reaction:

T4 DNA polymerase 3 U/μl 2 μl

The reactions were incubated for 30 minutes at 16°C.

The reactions were halted with EDTA, whereby the solution has the following composition:

20x EDTA/glycogen mix (200 mmol of EDTA, 1 mg/ml of glycogen) 4 μl

A phenol/chloroform extraction and an ethanol precipitation were performed for each reaction. The pellets were resuspended in 50 μl of water each.

### 1.5 Rsa I-Digestion of the ds cDNA

An Rsa I-digestion was performed both for the tester and for the driver. To this end, the following solutions were used:

ds cDNA 43.5 μl

10x Rsa I restriction buffer (100 mmol of bis tris propane-HCl, pH 7.0, 100 mmol of Mg-chloride, 1 mmol of DTT) 5 $\mu$l

Rsa I (10 U/$\mu$l) 1.5 $\mu$l

The reactions were incubated for 90 minutes at 37°C.

The reactions were then halted with EDTA, whereby the solution has the following composition:

20x EDTA/glycogen mix (200 mmol of EDTA 1 mg/ml of glycogen) 2.5 $\mu$l

Then, a phenol/chloroform extraction and an ethanol precipitation were performed for each reaction. The pellets that were produced in this connection were resuspended in 5.5 $\mu$l of water for further processing.

**1.6 Adaptor Ligation of ds Tester cDNA Digested on Rsa I**

The tester-cDNA was divided into 2 fractions. An adaptor was ligated to each tester fraction. The concentrations of the substances used for the two testers are cited in detail in the table below.

| | **Tester-1** | **Tester-2** |
|---|---|---|
| Tester-cDNA | 0.1 $\mu$l | 0.1 $\mu$l |
| 5x ligation buffer (250 mmol of tris-HCl, pH 7.8 50 mmol of $MgCl_2$ 100 mmol of DTT 0.25 mg/ml of BSA) | 2 $\mu$l | 2 $\mu$l |
| T4 DNA ligase (400 U/$\mu$l) | 1 $\mu$l | 1 $\mu$l |
| Adaptor 1 (10 $\mu$m) | 2 $\mu$l | -- |
| Adaptor 2 (10 $\mu$m) | -- | 2 $\mu$l |
| $H_2O$ | 4.9 $\mu$l | 4.9 $\mu$l |
| Total volumes | 10 $\mu$l | 10 $\mu$l |

The reactions were incubated overnight at 16°C and then halted with EDTA (20x EDTA/glycogen mix, 1 $\mu$l (200 mmol of EDTA, 1 mg/ml of glycogen)).

The reactions were incubated for 5 minutes at 72°C.

### 1.7 Subtractive Hybridizations

The driver and tester were then hybridized with one another in two steps.

**Hybridization**

The first hybridization was performed for the two reactions with the solutions and compounds that are cited in the table below:

| | Reaction 1 | Reaction 2 |
|---|---|---|
| Rsa-I-digested driver cDNA | 1.5 $\mu$l | 1.5 $\mu$l |
| Adaptor 1-ligated tester 1 | 1.5 $\mu$l | -- |
| Adaptor 2-ligated tester 2 | -- | 1.5 $\mu$l |
| 4x hybridization buffer | 1 $\mu$l | 1 $\mu$l |
| Total volumes | 4 $\mu$l | 4 $\mu$l |

The reactions were incubated for 90 seconds at 98°C and then directly for 8 hours at 68°C.

1. Hybridization

For the second hybridization, reactions 1 and 2 were mixed and freshly denaturated driver was added as follows:

| | |
|---|---|
| Driver | 1 $\mu l$ |
| 4x hybridization buffer | 1 $\mu l$ |
| water | 2 $\mu l$ |

1 $\mu l$ of this mixture was incubated for 90 seconds at 98°C and then fused as quickly as possible with reaction 1 and reaction 2.

The second hybridization was incubated overnight at 68°C. Then, 200 $\mu l$ of dilution buffer (20 mmol of HEPES-HCl (pH 8.3), 50 mmol of NaCl, 0.2 mmol of EDTA (pH 8.0)) was added to the second hybridization. Then, the second hybridization was incubated for 7 minutes at 68°C. The thus produced batch was then used for the PCR.

Differentially expressed fragments in the subtracted cDNA pools were selectively amplified by means of two successive PCRs.

The first PCR was performed with the following batch:

10x PCR buffer (400 mmol of tricine-KOH, pH 9.2, 150 mmol of KOAc,

| | |
|---|---|
| 35 mmol of MG(OAc)2, 37.5 $\mu g/ml$ of BSA) | 2.5 $\mu l$ |
| 10 mmol of dNTP | 0.5 $\mu l$ |
| PCR primer 1 (10 $\mu m$) | 1 $\mu l$ |
| 50x Advantage cDNA polymerase | 0.5 $\mu l$ |
| dilute second hybridization | 1 $\mu l$ |
| water | 19.5 $\mu l$ |

The PCR program was performed as follows: 75°C, 5 minutes
loop 94°C, 30 sec
66°C, 30 sec
72°C, 90 sec

Altogether, 27 cycles were performed.

The second PCR was performed with the following batch:

| | |
|---|---|
| 10x PCR buffer | 2.5 μl |
| 10 mmol of dNTP | 0.5 μl |
| nested PCR-primer 1 (10 μm) | 1 μl |
| nested PCR-primer 2R (10 μm) | 1 μl |
| 50x Advantage cDNA polymerase | 0.5 μl |
| PCR product | 0.1 μl |
| H2O | 19.4 μl |

The PCR program was performed as follows: 94°C, 30 seconds
68°C, 30 seconds
72°C, 90 seconds

Altogether, 12 cycles were performed.

The subtraction efficiency was checked by a semi-quantitative PCR for a known, unregulated gene (SH3P18). It showed a reduction in the subtracted cDNA pool by a factor of 150-200.

**2. Ligation of the Subtracted cDNA Pools in pUC 18**

The cDNA pools that were subtracted forwards and backwards were ligated in pUC 18 Sma I/BAP (SureClone Ligation Kit, Pharmacia Biotech) and subsequently cloned in chemically competent E. coli DH5$\alpha$.

To do this, the fragments of the subtracted cDNA pools were filled out until they formed blunt ends and were phosphorylated. The following compositions were used for this purpose:

| | | |
|---|---|---|
| Subtracted cDNA pool | | 1.59 $\mu$g |
| Klenow fragment | | 1 $\mu$l |
| 10x Blunting/kinasing buffer | | 2 $\mu$l |
| Polynucleotide kinase | | 1 $\mu$l |
| water | add | 20 $\mu$l. |

The reactions were incubated for 30 minutes at 37°C, then purified by way of PCR purification columns and eluted in 30 $\mu$l of water. Then, the DNA concentration was determined by means of OD-measurement.

## 2.1 Ligation in pUC 18

The ligation in pUC 18 was performed with the following batch:

| | | |
|---|---|---|
| Blunt-ended cDNA pool | | 50 ng |
| pUC 18 Sma I/BAP (50 ng/$\mu$l) | | 1 $\mu$l |
| 2x ligation buffer | | 10 $\mu$l |
| DTT | | 1 $\mu$l |
| T4 DNA ligase (6 U/$\mu$l) | | 3 $\mu$l |
| water | add | 20 $\mu$l |

The reactions were incubated overnight at room temperature.

## 2.2 Transformation of the Ligations in E. coli DH5$\alpha$

The ligations were transformed into chemically competent E. coli DH5$\alpha$. The transformed cells were streaked on 2YT agarose plates with 100 $\mu$g/ml of ampicillin, 625 $\mu$m of IPTG and 0.005% of X-Gal and cultured overnight at 37°C.

A colony-PCR with vector-primers (M13 standard primer) was performed on 17 randomly selected white clones. In this case, 15-16 clones showed inserts with a size distribution that corresponded to that of the cDNA pool used.

For each subtraction, 1536 clones in 384-well plates were transferred with 50 $\mu$l of 2YT, 1xHMFM, and 100 $\mu$g/ml of ampicillin per well. The filled 384-well plates were incubated overnight at 37°C and could then be stored at -80°C.

**3. Production of Colony Filters:**

The 1536 clones of a subtractive cDNA-bank were inoculated on a Hybond Nylon N+ membrane (Amersham). The membrane was placed on a 2YT agarose plate with 100 $\mu$g/ml of ampicillin and incubated overnight at 37°C. The membrane was placed with the colony side upward for 4 minutes on Whatman 3MM paper soaked with denaturation solution (0.5M NaOH, 1.5M NaCl). Then, the membrane was incubated for 4 minutes on Whatman 3MM paper soaked in neutralization solution (1 M tris-HCl (pH 7.5), 1.5M NaCl). The membrane was then treated for 1 hour at 37°C with proteinase K. The membrane was immersed to this end in 300 ml of proteinase K buffer (50 mmol of NaCl, 5 mmol of EDTA, 10 mmol of tris-HCl (pH 8), 50 mg/ml of proteinase K). Finally, the membrane was dried at 80°C for 3 hours and was then used for the hybridizations.

**4. Differential Hybridization:**

To identify the differential expression of the cloned fragments, a differential hybridization on colony-filters of subtractive cDNA-banks was performed with the aid of a PCR-select differential screening kit.

To ensure specific hybridization of the forwards- and backwards-subtracted cDNA pools onto the subtractive cDNA-bank colony filter, it was necessary to remove the adaptor sequences in the hybridization sample.

- As hybridization samples for the Rsa I-restriction, the subtracted cDNA pools were used:

| | |
|---|---|
| cDNA pool | 28 $\mu$l |
| 10x Rsa I restriction buffer (100 mmol of bis tris propane-HCl, pH 7.0, 100 mmol of Mg-chloride, 1 mmol of DTT) | 3 $\mu$l |
| Rsa I (10 U/$\mu$l) | 2 $\mu$l |

The reactions were incubated at 37°C for 5 hours and then purified on PCR-purification columns and eluted in 30 $\mu$l of water. The DNA concentration was determined by means of OD measurement.

**5. Radioactive Labeling of the Subtracted cDNA Pools**

The radioactive labeling of the subtracted cDNA pools was performed with the following batch:

| | | |
|---|---|---|
| cDNA pool | 150 ng in | 9 $\mu$l |
| reaction buffer, -dCTP (333 mmol of tris-HCl, pH 8, 33.3 Mg-chloride, 10 mmol of 2-mercaptoethanol, 170 $\mu$m of dATP, 170 $\mu$m of dGTP, 170 $\mu$m of dTTP) | | 3 $\mu$l |
| random primer mix (0.9 mg/ml of random nonamers, 50 mmol of tris-HCl, pH 7.5, 10 mmol of Mg-chloride, 1 mmol of DTT, 50 $\mu$g/ml of BSA) | | 2 $\mu$l |
| AP32 dCTP | | 3 $\mu$l |
| Klenow fragment (3 U/$\mu$l) | | 1.5 $\mu$l |

The reactions were incubated at 37°C for 1 hour, then purified on PCR-purification columns and eluted in 30 $\mu l$ of water. The specific activity of the reactions was determined to ensure that in both hybridization reactions, the same amount of labeled DNA was used.

**6. Prehybridization and Hybridization of Filters and Hybridization Samples**

For the hybridizations, the following solution was used:

| | |
|---|---|
| 20x SSC | 50 $\mu l$ |
| Blocking solution (10 mg/ml of sheared salmon sperm DNA, 0.3 mg/ml of complementary Oligos to the adaptors) | 50 $\mu l$ |

The solution was incubated for 5 minutes at 98°C, then put on ice for 5 minutes and mixed with 5 ml of express-hybridization solution. This solution was then prehybridized in the hybridization flask with the filter at 72°C for 1 hour.

The hybridization samples were also mixed with the following solution:

| | |
|---|---|
| 20x SSC | 50 $\mu l$ |
| Blocking solution (10 mg/ml of sheared salmon sperm DNA, 0.3 mg/ml of complementary oligos to the adaptors) | 50 $\mu l$ |

The batch was then incubated for 5 minutes at 98°C and for 2 minutes on ice. The hybridization samples were then added to the

filter in the hybridization flasks and hybridized overnight at 72°C.

Then, the procedure was as follows:

a) 4 x 20 minutes at 68°C with preheated 2xSSC, 0.5% SDS

b) 2 x 20 minutes at 68°C with preheated 0.2xSSC, 0.5% SDS

c) then exposure in phosphorus-imager-cassettes for 22 hours at room temperature.

**7. Evaluation of Differential Hybridizations**

The evaluation of the hybridizations was carried out on a phosphorus imager.

A clone was then classified as differentially expressed if it showed only a detectable hybridization signal with the forwards-subtracted cDNA pool or if the signal strength with the forwards-subtracted cDNA pool was larger by at least the factor of 5 than with the backwards-subtracted cDNA pool.

**8. Confirmation of the Differential Expression by Means of Semi-quantitative RT-PCR**

To confirm the differential expression of the clones with a differential hybridization result, sequences were selected randomly, and corresponding primers were produced.

As a method for detecting the differential expression, the comparative multiplex RT-PCR according to Pilarsky et al. (The Prostate 36: 85-91 (1998)) was used. As an internal standard, primers of the 23kD highly basic protein were used. The sequence of interest and the standard fragment were amplified

simultaneously in a reaction for a different number of cycles. The PCR products were then separated on a 6% sequencer gel and analyzed by means of software and quantified. First, the number of cycles was determined for which both the standard fragment and the sequence of interest were linearly amplified and which then were used for the quantifying PCR. For quantifying RT-PCR, different RNA preparations were used and in each case 3 reactions were prepared.

For 90% of the sequences examined with a differential hybridization result, a difference in the expression that was greater than a factor of 2 could be noted.



**9. Automatic Extension of the Nucleic Acid Sequences Found**

To obtain as much sequence information as possible for each differentially expressed clone, an automatic extension of the starting sequence based on all available EST sequences was performed.

The automatic extension of sequence S takes place in three steps:

1. Determination of all S-homologous sequences from the total amount of all available ESTs from the LifeSeq database (status as of October 1997) with the aid of the BLAST algorithm (Altschul, S.; Gish, W.; Miller, W.; Myers, E.; Lipman, D. (1990) **J. Mol. Biol.**, 215, 403-410).

2. Assembling of these sequences by means of the standard program GAP4 (Bonfield, J.; Smith, K.; Staden, R. (1995), **Nucleic Acids Research** 23, 4992-4999).

3. Calculation of a consensus sequence from the assembled sequences.

An attempt is now made to extend the consensus sequence in the same way. This iteration is continued with the consensus sequence that is obtained in each case, until no further extension is possible.

**10. Nucleic Acid Sequences that are Found**

Analogously to the procedure that is described under 1 to 9, e.g., the following sequences were found, of which several are over-expressed in culture type a) or culture type b) of the endothelial cells.

These nucleic acid sequences are also the subject matter of this invention.

Angiogenesis relates to the possible function of these gene areas.

The result is depicted in Table I below:

**TABLE I**

| Seq ID No. | Expression | Function | Homology |
|---|---|---|---|
| 1 | Over-expressed in a) | Associated with proliferation | None |
| 2 | Over-expressed in a) | Associated with proliferation | None |
| 3 | Over-expressed in b) | Associated with differentiation | None |
| 4 | Over-expressed three times in b) | Gap junction, associated with differentiation | Connexin37; 96% identity over 933 bp |
| 5 | Over-expressed in a) | Associated with proliferation | None |
| 6 | Over-expressed twice in b) | Associated with differentiation | None |
| 7 | Over-expressed in a) | Associated with proliferation | None |
| 8 | Over-expressed in b) | Associated with differentiation | None |
| 9 | Over-expressed in b) | Associated with differentiation | None |
| 10 | Over-expressed in b) | Associated with differentiation | SPRY2; 99% identity over 1489 bp |
| 11 | Over-expressed in b) | Associated with differentiation | None |
| 12 | Over-expressed in b) | Associated with differentiation | Mouse Gas5; 78% identity over 121 bp |
| 13 | Over-expressed in b) | Associated with differentiation | None |
| 14 | Over-expressed in b) | Associated with differentiation | None |
| 15 | Over-expressed in b) | Associated with differentiation | None |

| | | | |
|---|---|---|---|
| 16 | Over-expressed in b) | Associated with differentiation | None |
| 17 | Over-expressed in b) | Associated with differentiation | None |
| 18 | Over-expressed in b) | Associated with differentiation | None |
| 19 | Over-expressed in b) | Associated with differentiation | None |
| 20 | Over-expressed in b) | Associated with differentiation | None |
| 21 | Over-expressed in b) | Associated with differentiation | None |

| Seq ID No. | Expression | Function | Homology |
|---|---|---|---|
| 22 | Over-expressed in b) | Associated with differentiation | None |
| 23 | Over-expressed five times in b) | Associated with differentiation | Mouse MMP; 83% identity over 831 bp |
| 24 | Over-expressed in b) | Associated with differentiation | None |
| 25 | Over-expressed four times in b) | Associated with differentiation | None |
| 26 | Over-expressed in b) | Associated with differentiation | None |
| 27 | Over-expressed in b) | Associated with differentiation | None |
| 28 | Over-expressed in b) | Associated with differentiation | KIAA0255; 57% identity over 326 bp |
| 29 | Over-expressed in b) | Associated with differentiation | Thymic epithelial cell antigen; 68% identity over 326 bp |
| 30 | Over-expressed in b) | Associated with differentiation | None |
| 31 | Over-expressed four times in b) | Associated with differentiation | None |
| 32 | Over-expressed in b) | Associated with differentiation | None |
| 33 | Over-expressed in b) | Associated with differentiation | None |
| 34 | Over-expressed in b) | Associated with differentiation | None |
| 35 | Over-expressed in b) | Associated with differentiation | None |

| | | | |
|---|---|---|---|
| 36 | Over-expressed in a) | Associated with proliferation | None |
| 37 | Over-expressed in b) | Associated with differentiation | CL-20; 87% identity with 122 bp |
| 38 | Over-expressed five times in b) | Associated with differentiation | Mouse numb; 90% identity over 310 bp |
| 39 | Over-expressed in a) | Associated with proliferation | None |
| 40 | Over-expressed in b) | Associated with differentiation | None |
| 41 | Over-expressed five times in a) | Associated with proliferation | None |
| 42 | Over-expressed six times in a) | Coreprocessor, associated with proliferation | SMRT; 99% identity over 785 bp |
| 43 | Over-expressed in a) | Associated with proliferation | None |
| 44 | Over-expressed in a) | Associated with proliferation | None |
| 45 | Over-expressed in a) | Associated with proliferation | None |
| 46 | Over-expressed in a) | Associated with proliferation | None |

| Seq ID No. | Expression | Function | Homology |
|---|---|---|---|
| 47 | Over-expressed five times in b) | Associated with differentiation | None |
| 48 | Over-expressed in a) | Associated with proliferation | MUC18; 99% identity over 780 bp |
| 49 | Over-expressed in a) | Associated with proliferation | None |
| 50 | Over-expressed in a) | Associated with proliferation | None |
| 51 | Over-expressed three times in a) | Associated with proliferation | None |
| 52 | Over-expressed in a) | Associated with proliferation | None |
| 53 | Over-expressed in a) | Associated with proliferation | None |
| 54 | Over-expressed in a) | Associated with proliferation | None |
| 55 | Over-expressed seven times in a) | Associated with EC proliferation and migration | CYR61; 100% identity over 2015 bp |
| 56 | Over-expressed in a) | Associated with proliferation | None |
| 57 | Over-expressed in a) | Associated with proliferation | None |
| 58 | Over-expressed three times in a) | Associated with proliferation | None |
| 59 | Over-expressed in b) | Associated with differentiation | None |

a), b) = culture types

11. **Expression Analysis**

To examine whether the regulated sequences are also involved **in vivo** in the formation of new blood vessels, their expression in human placenta tissue in the eighth week was found to have a high angiogenesis activity, and their expression in human placenta tissue in the ninth month was found to have little angiogenesis activity. A stronger expression in the 8-week placenta was in this case evaluated as a reference to an angiogenesis-relevant function of the sequence. A stronger expression in the 9-month-old placenta was considered as a reference to a vessel-stabilizing function of the sequence. To this end, a semi-quantitative RT-PCR technique was used, the comparative multiplex RT-PCR. In this method, the expression of the sequence of interest becomes relative to the expression of a non-differentially regulated so-called "household gene," here the 23kD highly basic protein. As a positive control, the expression of the VEGF receptor KDR was determined. Of this endothelial cell-specific gene, it is known that it is highly regulated on angiogenetically active endothelium. A significantly increased KDR-expression in the 8-week placenta was correspondingly detected in comparison to the 9-month-old placenta.

The results are summarized in Table II:

Table II

| Sequence | MVEC, proliferating | 8-Week placenta | 9-Month placenta |
|---|---|---|---|
| 1 | *** | *** | - |
| 2 | n.d. | | |
| 3 | **** | *** | * |
| 4 | ** | ** | ** |
| 5 | *** | *** | * |
| 6 | *** | ** | ** |
| 7 | * | **** | ** |
| 8 | n.d. | | |
| 9 | ** | * | *** |
| 10 | *** | **** | * |
| 11 | ** | - | ** |
| 12 | n.d. | | |
| 13 | - | ** | - |
| 14 | **** | *** | * |
| 15 | *** | ** | - |
| 16 | - | - | - |
| 17 | *** | ** | - |
| 18 | **** | *** | - |
| 19 | n.d. | | |
| 20 | *** | **** | ** |
| 21 | n.d. | | |
| 22 | ** | ** | ** |
| 23 | * | **** | ** |
| 24 | *** | ** | - |
| 25 | ** | * | * |
| 26 | n.d. | | |
| 27 | ** | * | * |
| 28 | ** | * | - |
| 29 | * | * | * |
| 30 | **** | *** | - |
| 31 | ** | *** | *** |
| 32 | **** | *** | * |
| 33 | *** | ** | ** |
| 34 | ** | ** | **** |
| 35 | * | - | - |
| 36 | **** | *** | - |
| 37 | n.d. | | |
| 38 | *** | ** | - |
| 39 | *** | **** | ** |
| 40 | ** | - | - |
| 41 | *** | *** | * |
| 42 | ** | * | * |
| 43 | n.d. | | |
| 44 | *** | *** | *** |
| 45 | n.d. | | |
| 46 | *** | * | * |
| 47 | n.d. | | |
| 48 | *** | ** | - |

| | | | |
|---|---|---|---|
| 49 | ** | - | - |
| 50 | n.d. | | |
| 51 | ** | ** | ** |
| 52 | *** | **** | ** |
| 53 | **** | **** | ** |
| 54 | n.d. | | |
| 55 | ** | ** | **** |
| 56 | *** | *** | * |
| 57 | **** | *** | - |
| 58 | **** | ** | - |
| KDR | ** | **** | ** |

Key to the table:

**** = very strong expression

*** = strong expression

** = moderate expression

* = weak expression

- = expression below the detection limits

n.d. = not performed

The nucleic acid sequences Seq. ID No. 1 to Seq. ID No. 59 of the determined candidate genes according to the invention are described in the sequence protocol below.

Based on the considerable over-expression of sequence 34 in the tubular MVEC (>8x) and a weak homology to thrombospondin-2, a gene, which plays an important role in the maturation of the blood vessel system, sequence 34 was selected from the wide variety of sequences for further analysis. Starting from the identified partial sequence, the complete mRNA sequence for sequence 34 was determined by means of 5'- and 3'-RACE experiments. With a length of 6011 bp, the size of sequence 34 corresponds very well to the size (-6kb) determined in a Northern

hybridization. The complete mRNA sequence contains an open reader frame that codes for 1036 amino acids. The referenced protein has a molecular weight of -114kD, is cysteine-rich (12.5% cysteine content) and has a domain structure that has been unique up until now. The protein has an N-terminal signal peptide, a portion of a thiol protease domain, an RGD-pattern, 6 Von-Willebrand-factor type C-domains, a potential transmembrane domain and 5 possible N-glycosylation points. In addition, the genomic localization of sequence 34 in Chr. 2p21 and the complete Intron/Exon structure were determined.

Based on the domain structure of the protein, a type 1 transmembrane orientation can be assumed, with a long extracellular N-terminus and a short intracellular C-terminus. To test this, a rabbit-antiserum was produced, which is oriented against a peptide from the extracellular portion of the protein. With the aid of this antiserum, it was possible to show that the protein actually has a type I-transmembrane orientation.

This anti-sequence 34-serum was used for immunohistological studies in sections of an ovarian carcinoma, or a prepuce. In this case, it was shown that sequence 34 in the tumor is expressed from endothelial cells, but not from stromal cells. No sequence 34-expression could be detected in the prepuce, however. Sequence 34 is thus expressed in the angiogenetically active tumor endothelium of the ovarian carcinoma that is studied, but not in the dormant endothelium of the normal tissue. These results were confirmed by **in situ** hybridizations on the mRNA plane.

To determine the expression profile for sequence 34, a Northern hybridization was performed on various human tissues. In this case, an expression pattern for sequence 34, which suggests a specific function of the protein in endothelial cells, was shown with the strongest expression in the placenta, followed by the kidney, the heart and the lung.

To test whether sequence 34 has an important function in the tubulus formation in the **in vitro** model on matrigel, antisense oligonucleotides were produced. It was possible to determine an oligonucleotide that inhibits the sequence 34 expression. This oligonucleotide was not toxic for the cells and did not result in an altered proliferation behavior of the treated cells. Endothelium cells, which were transfixed with this oligonucleotide, showed, however, a dramatic inhibition of the tubulus formation on matrigel (> 20% of the control value) in comparison to untransfixed cells and cells transfixed with a control oligonucleotide. Sequence 34 thus contributes significantly to the formation of capillary-like structures. These results are consistent with the data from the expression analysis in the two placenta samples for sequence 34. The stronger expression 34 in the 9-month-old placenta was evaluated as a reference to a vessel-stabilizing function of the sequence. The antisense-oligonucleotide data clearly show that sequence 34 does not play any role during the endothelial cell proliferation but is involved significantly in the formation of stable capillary structures.

The invention thus relates in particular to the sequence Seq ID No. 34 and its use for the formation of stable capillary structures. In addition, this sequence and the protein sequence derived therefrom also relate to the use, either alone or in a formulation as a pharmaceutical agent for treatment of psoriasis, arthritis, such as rheumatoid arthritis, hemangioma, angiofibroma, eye diseases, such as diabetic retinopathy, neovascular glaucoma, nephropathies, such as glomerulonephritis, diabetic nephropathy, malignant nephrosclerosis, thrombic microangiopathic syndrome, transplant rejections and glomerulopathy, fibrotic diseases, such as cirrhosis of the liver, mesangial cell proliferative diseases, arteriosclerosis and injuries to the nerve tissue.

**Claims**

1. A nucleic acid sequence that codes a gene product or a portion thereof, comprising

a) a nucleic acid sequence that is selected from the group of Seq. ID No. 1 to Seq. ID No. 59

b) an allelic variation of the nucleic acid sequences named under a)

or

c) a nucleic acid sequence that is complementary to the nucleic acid sequences named under a) or b).

2. A nucleic acid sequence according to one of the sequences Seq. ID No. 1 to Seq. ID No. 59 or a complementary or allelic variant thereof.

3. Nucleic acid sequences Seq. ID No. 1 to Seq. ID No. 59, characterized in that it is expressed elevated in endothelial cell tissue.

4. BAC, PAC and Cosmid clones containing functional genes and their chromosomal localization according to nucleic acid sequences Seq. ID No. 1 to Seq. ID No. 59 for use as a vehicle for gene transfer.

5. A nucleic acid sequence according to claims 1 to 4, wherein it has 90% homology to a human nucleic acid sequence.

6. A nucleic acid sequence according to claims 1 to 4, wherein it has 95% homology to a human nucleic acid sequence.

7. A nucleic acid sequence comprising a portion of the nucleic acid sequences named in claims 1 to 6, in such a

sufficient amount that they hybridize with the sequences according to claims 1 to 6.

8. A nucleic acid sequence according to claims 1 to 7, wherein the size of the fragment has a length of at least 50 to 3000 bp.

9. A nucleic acid sequence according to claims 1 to 7, wherein the size of the fragment has a length of at least 150 to 2800 bp.

10. A nucleic acid sequence according to claims 1 to 7, wherein the size of the fragment has a length of at least 150 to 2600 bp.

11. A nucleic acid sequence according to one of claims 1 to 10, which codes at least one partial sequence of a bioactive polypeptide.

12. An expression cassette, comprising a nucleic acid fragment or a sequence according to one of claims 1 to 10, together with at least one control or regulatory sequence.

13. An expression cassette, comprising a nucleic acid fragment or a sequence according to claim 12, in which the control or regulatory sequence is a suitable promoter.

14. An expression cassette according to one of claims 12 and 13, wherein the DNA sequences located on the cassette code a fusion protein, which comprises a known protein and a bioactive polypeptide fragment.

15. Use of nucleic acid sequences according to claims 1 to 11 for producing full-length genes.

16. A DNA fragment, comprising a gene, that can be obtained from the use according to claim 15.

17. Host cell, containing as the heterologous part of its expressible genetic information a nucleic acid fragment according to one of claims 1 to 11.

18. Host cell according to claim 17, wherein it is a prokaryotic or eukaryotic cell system.

19. Host cell according to one of claims 17 or 18, wherein the prokaryotic cell system is E. coli, and the eukaryotic cell system is an animal, human or yeast cell system.

20. A process for the production of a polypeptide or a fragment, wherein the host cells according to claims 17 to 19 are cultivated.

21. An antibody that is directed against a polypeptide or a fragment that is coded by the nucleic acids of sequences Seq. ID No. 1 to Seq. ID No. 59, which can be obtained according to claim 20.

22. An antibody according to claim 21, wherein it is monoclonal.

23. Polypeptide sequence, expressed by one of nucleic acid sequences Seq. ID No. 1 to Seq. ID No. 59.

24. Polypeptide sequences according to claim 23, with at least 80% homology to these sequences.

25. Polypeptide sequences according to claim 23, with at least 90% homology to these sequences.

26. Polypeptide sequence, wherein it comprises the sequence Seq ID No. 34.

27. Use of polypeptide sequences according to claims 23 to 26 as tools for finding active ingredients against angiogenetic diseases.

28. Use of nucleic acid sequences according to sequences Seq. ID No. 1 to Seq. ID No. 59 for expression of polypeptides that can be used as tools for finding active ingredients against angiogenetic diseases.

29. Use of nucleic acid sequences Seq. ID No. 1 to Seq. ID No. 59 in sense or antisense form.

30. Use of polypeptide sequences according to claims 23 to 26 as pharmaceutical agents in gene therapy for treatment of angiogenetic diseases.

31. Use of polypeptide sequences according to claims 23 to 26 for the production of a pharmaceutical agent for treatment of angiogenetic diseases.

32. Pharmaceutical agent, containing at least one polypeptide sequence according to claims 23 to 26.

33. A nucleic acid sequence according to claims 1 to 11, wherein it is a genomic sequence.

34. A nucleic acid sequence according to claims 1 to 11, wherein it is an mRNA sequence.

35. Genomic genes, their promoters, enhancers, silencers, Exon structure, Intron structure and their splice variants, that can be obtained from cDNAs of sequences Seq. ID No. 1 to Seq. ID No. 59.

36. Use of the genomic genes according to claim 35, together with suitable regulatory elements.

37. Use according to claim 36, wherein the regulatory element is a suitable promoter and/or enhancer.

38. Use of the nucleic acid sequences according to claims 1 to 11 and the peptides according to claims 23 to 26, either alone or in a formulation as a pharmaceutical agent for treatment of psoriasis, arthritis, such as rheumatoid arthritis, hemangioma, angiofibroma, eye diseases, such as diabetic retinopathy, neovascular glaucoma, nephropathies, such as glomerulonephritis, diabetic nephropathy, malignant nephrosclerosis, thrombic microangiopathic syndrome, transplant rejections and glomerulopathy, fibrotic diseases, such as cirrhosis of the liver, mesangial cell proliferative diseases, arteriosclerosis and injuries of the nerve tissue.

39. Nucleic acid sequence Seq. ID No. 34, wherein it forms stable capillary structures.

40. Use of nucleic acid sequence Seq. ID No. 34 and the peptides expressed via this sequence, either alone or in a formulation as a pharmaceutical agent for treatment of psoriasis, arthritis, such as rheumatoid arthritis, hemangioma, angiofibroma, eye diseases, such as diabetic retinopathy, neovascular glaucoma, nephropathies, such as glomerulonephritis, diabetic nephropathy, malignant nephrosclerosis, thrombic microangiopathic syndrome, transplant rejections and glomerulopathy, fibrotic diseases, such as cirrhosis of the liver, mesangial cell proliferative diseases, arteriosclerosis and injuries of the nerve tissue.

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**Veröffentlicht**
*Ohne internationalen Recherchenbericht und erneut zu veröffentlichen nach Erhalt des Berichts.*

## (54) Title: HUMAN NUCLEIC ACID AND PROTEIN SEQUENCES OBTAINED FROM ENDOTHELIAL CELLS

## (54) Bezeichnung: MENSCHLICHE NUKLEINSÄURE- UND PROTEIN-SEQUENZEN AUS ENDOTHELZELLEN

**(57) Abstract**

The invention relates to nucleic acid sequences – mRNA, cDNA, genome sequences – obtained from human endothelial cells and coding for gene products or parts thereof, as well as to their use. The invention also relates to the polypeptides obtained by means of said sequences and to their use.

**(57) Zusammenfassung**

Es werden Nukleinsäure-Sequenzen – mRNA, cDNA, genomische Sequenzen – aus Gewebe menschlicher Endothelzellen, die für Genprodukte oder Teile davon kodieren und deren Verwendung, beschrieben. Es werden weiterhin die über die Sequenzen erhältlichen Polypeptide und deren Verwendung beschrieben.

Attorney Docket Number: SCH 1821

# DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**HUMAN NUCLEIC ACID SEQUENCES AND PROTEIN SEQUENCES FROM ENDOTHELIAL CELLS**

the specification of which

☐ is attached hereto

■ was filed on 8 MARCH 2000 as United States Application Number or PCT International Application Number PCT/EP00/02005 and (if applicable) was amended on ____________

I hereby authorize our attorneys to insert the serial number assigned to this application.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

| PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 USC §119 | | | |
|---|---|---|---|
| APPLICATION NO. | COUNTRY | DAY/MONTH/YEAR FILED | PRIORITY CLAIMED |
| 199 11 684.9 | GERMANY | 9 MARCH 1999 | YES |
| 199 48 679.4 | GERMANY | 1 OCTOBER 1999 | YES |

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

| PROVISIONAL APPLICATION(S) UNDER 35 U.S.C. §119(e) | |
|---|---|
| APPLICATION NUMBER | FILING DATE |
| | |

I hereby claim the benefit under 35 U.S.C. §120 of any United States application, or §365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. §112.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

| PRIOR U.S./PCT INTERNATIONAL APPLICATION(S) DESIGNATED FOR BENEFIT UNDER 37 U.S.C. §120 | | |
|---|---|---|
| APPLICATION NO. | FILING DATE | STATUS — PATENTED, PENDING, ABANDONED |
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I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith: I. William Millen (19,544); John L. White (17,746); Anthony J. Zelano (27,969); Alan E.J. Branigan (20,565); John R. Moses (24,983); Harry B. Shubin (32,004); Brion P. Heaney (32,542); Richard J. Traverso (30,595); John A. Sopp (33,103); Richard M. Lebovitz (37,067); John H. Thomas (33,460); Catherine M. Joyce (40,668); Nancy J. Axelrod (44,014); James T. Moore (35,619); James E. Ruland (37,432); Jennifer J. Branigan (40,921) and Robert E. McCarthy (46,044)

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☐ Additional joint inventors are named on separately numbered sheets attached hereto.